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# High mobility group proteins 1 and 2 are present in simian virus 40 provirions, but not in virions

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## ABSTRACT

Viral particles at the late stages of SV40 morphogenesis were examined for the presence of HMG proteins 1 and 2, by an immunochemical method involving the transfer of proteins from polyacrylamide gels to nitrocellulose membranes. It was found that these proteins are present in SV40 provirions, in which histone H1 is still associated with viral chromatin, but absent in mature SV40 virions.

## INTRODUCTION

It is well established that SV40 chromatin is arranged in a structure which is morphologically similar to that of eukaryotic cell chromatin (1-4). Among the non-histone proteins which are found in association with the cell chromatin, the high mobility group (HMG) proteins are a well-characterized family of special interest, since it has been suggested that some of them may play a structural role in the organization of the genomic material (5-7), while others may be involved in mechanisms controlling gene expression (8-10). In the present study, we investigated whether HMG 1 and 2 are present in SV40 chromatin at some or all of the stages of virus growth. In a previous report we had shown that infected CV-1 cells contained a population of immature viral particles, called provirions, which differed from the morphologically similar mature virions because they were less stable and still contained histone H1 (11). On the other hand, the histones in these provirions already possess the high degree of acetylation typical of virion chromatin. Our attention has been focused here on the SV40 provirions, since they presumably constitute a transitional stage between a viral chromatin still conserving many host-like properties and the final arrangement of chromatin in stable, mature particles. Our results show that HMG-1 and HMG-2 proteins are present in SV40 provirions, whereas in virions, as it happens for histone H1, they are either absent or below the level of detection of our very sensitive radioimmunological method.

## MATERIALS AND METHODS

### Cell culture and virus

CV-1 cells were grown to confluence in 10-cm plastic dishes and infected at a multiplicity of 5 to 10 with a large-plaque clone of SV40 as previously described (11).

### Isolation of SV40 virions and provirions

The procedure to isolate the viral particles followed that already reported in detail (11). Briefly, mature virions were collected at the end of the complete lysis of infected cultures, which occurred around 120 hr post-infection; the particles were sedimented by differential centrifugation from the 10,000 x g supernatant of the lysate, then resuspended and centrifuged in sucrose gradients similar to those used for cell extracts (see below). Provirions were extracted from infected cultures at 60 to 65 hr post-infection; cells harvested by scraping were swollen for 5 min in a hypotonic buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 0.1 mM EDTA, 0.3 mg/ml PMSF) and disrupted in a Dounce homogenizer. The extracts, brought to 1 mM EDTA, were layered on a 15-30% sucrose gradient in 10 mM Tris, pH 7.4, 100 mM NaCl, and centrifuged for 100 min at 40,000 rpm in a Spinco SW 41 rotor. The peak sedimenting at 180 S (with reference to the sedimentation rate of ribosomal subunits, see Fig. 3) was precipitated by the addition of 2 volumes of ethanol.

In other experiments, CV-1 cells were labelled for 12 hr with $^{3}$H-leucine (NEN) (50 Ci/mmol) at a concentration of 10 µCi/ml. At the end of the labelling period, the cells were collected and mixed with an equal amount of SV40 infected CV-1 cells and the cell mixture was then treated as above. In parallel experiments, 0.5 µg of $^{125}$I-labelled HMG-1 and HMG-2 (spec. act. 5 x $10^{7}$ cpm/mg) was added to 2 x $10^{7}$ cells before homogenization.

### HMG and nucleosome preparation

HMG chromosomal proteins were extracted from calf thymus nuclei with 0.35 M sodium chloride and purified to homeogeneity as previously described (5). Calf thymus nucleosomes were prepared by micrococcal nuclease digestion and fractionated on a 5-20% sucrose gradient as previously described (8). HMG proteins were labelled with 100 µCi of $^{125}$I using a modification of the chloramine T method (12,13).

### Anti-HMG antisera

Rabbit anti-HMG-2 sera were prepared as previously described (14).

### Radioimmunoassay

The solid phase radioimmunoassay method used was previously described (13).

Gel electrophoresis and protein-transfer technique

Samples were dissolved in 10 mM sodium phosphate, pH 8.4, 1% SDS, 4 mM DDT, heated in a boiling bath for 1 min, and electrophoreses for 15 hr at 10 mA in a 15% polyacrylamide slab gel according to Laemmli (15). Proteins were electrophoretically transferred from the gel to a nitrocellulose membrane as described by Towbin et al. (16), using a gel destained (EC Apparatus) for 1.5 hr at 15 V, 800 mA. After transfer, the membrane strip was soaked for 1 hr in 150 mM NaCl, 10 mM Tris, pH 7.4, 3% BSA, 10% fetal bovine serum, 0.01% $NaN_3$. The membrane was then incubated with either the anti-serum or the normal rabbit serum diluted 1:100 in 150 mM NaCl, 10 mM Tris, pH 7.4, 1% BSA, 0.01% $NaN_3$ (100 µl per $cm^2$) for 15 hr at room temperature with gentle shaking. The membrane, washed with 100 ml of the same buffer, was then incubated for 1 hr with 25,000 cpm/$cm^2$ of staphylococcus aureus protein A labelled with $^{125}I$ as described before (12,13), washed again 3-5 hr, blotted dry, and autoradiographed for 1-24 hr with Cronex HI plus intensifier screen at -80°C.

Limited proteolytic digestion of HMG and VP3

HMG and VP3 proteins used for proteolytic digestion experiments were purified by a preparative gel electrophoresis method. Gels were rapidly stained and destained, the bands corresponding to VP3 and HMG were cut out, and the protein-containing strips were processed as described by Cleveland et al.(17), using V 8 protease as the proteolytic enzyme. Peptides were then transferred from the gel to the nitrocellulose filter and processed as described above.

## RESULTS

Specificity of the antiserum

In previous papers we have shown, by microcomplement fixation and solid-phase radioimmunoassay, that our antisera are able to recognize HMG-1 and HMG-2 proteins and that no cross-reactivity is detectable with histones. HMG-1 and HMG-2 are strongly cross-reacting proteins (12,13), presumably because their amino acid sequences are very similar (19). Electrophoresis in polyacrylamide gels containing SDS and urea is supposed to alter considerably the conformation of proteins. Since this phenomenon (followed by the protein transfer to nitrocellulose in non-denaturing buffer) could result in a change of immunological behavior, experiments were carried out to test the antisera specificity under these new conditions. Purified HMGs, histones, and nucleosomes were electrophoretically transferred from SDS gels to nitrocellulose membranes, then reacted with the anti-HMG serum. Figures 1 and 2 show the reaction against HMG-1, HMG-2, nucleosome proteins, and H1. The only bands

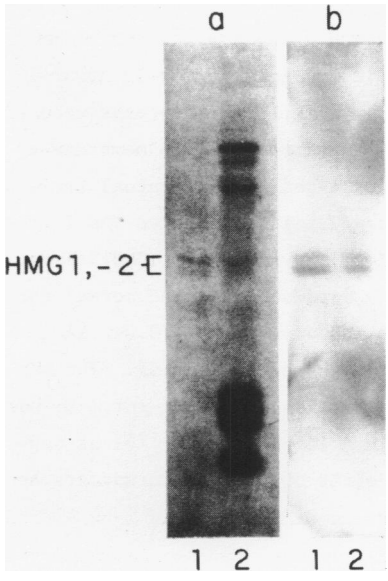


Figure 1. Specificity of antiserum directed against HMG-1 and HMG-2 proteins.
a) SDS polyacrylamide gel. 1: Calf thymus HMG-1, HMG-2. 2: Calf thymus nucleosomes.
b) Autoradiography of the transfer stained with the antisera.

detected upon autoradiography correspond to HMG proteins 1 and 2; no reaction is seen with the core histones and H1.

Identification of HMG-1 and HMG-2 in SV40 provirions

The immature form of SV40 virions (provirions) as well as mature virions were isolated by the procedures indicated under Methods. The total proteins

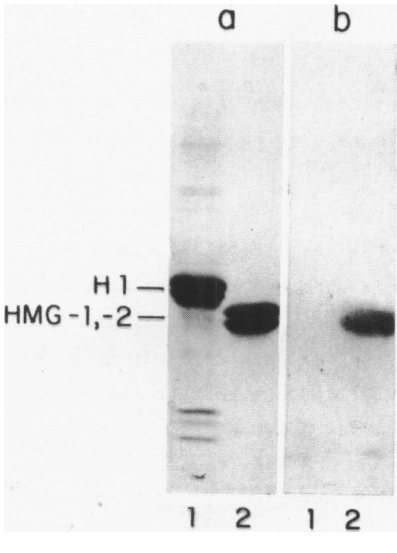


Figure 2. Specificity of antiserum directed against HMG-1 and HMG-2 proteins.
a) SDS polyacrylamide gel. 1: Calf thymus histone H1. 2: Calf thymus HMG-1, HMG-2
b) Autoradiography of the gel transfer stained with the antisera

from the two types of SV40 particles were separated on SDS polyacrylamide gel and then transferred to a nitrocellulose membrane in which they were detected by immunological technique (see Methods). Figure 3, panel a, shows the electrophoretic pattern of the proteins from the immature and mature virions after staining with Coomassie Blue. The same figure, panel b, shows these proteins after their transfer to the nitrocellulose membrane and the reaction with the anti-HMG-2 antisera.

The results indicate that 1) HMG-1 and HMG-2 are present in SV40 provirions, 2) HMG-1 and HMG-2 are absent in the mature virions, and 3) VP3 is also recognized by the antisera. The lower band detected in lane 2 is not due to a reaction of the antiserum with the histone H3 because experiments run with purified H3 and other histones were all negative (data not shown); most likely, this band is a degradation product of the HMGs.

Similar experiments were conducted with the 75 S "minichromosomes." Our results indicate that HMG-1 and -2 are present in this structure; however, they are extensively degradated and are detected as fragments in our immunoassay

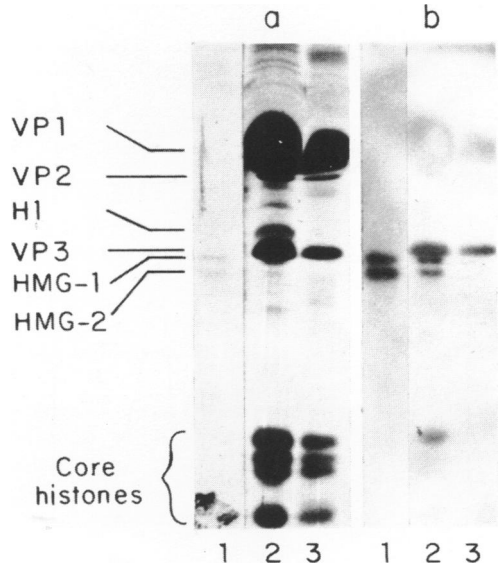


Figure 3. Electropherograms and transfer pattern of total proteins from SV40 provirions and mature virions. The two types of particles were prepared as described in Methods.

a) SDS polyacrylamide gel. 1: Calf thymus HMG-1, HMG-2. 2: Proteins from provirions. 3: Proteins from mature virions.

b) Autoradiography of the transfer stained with anti-HMG-2 antiserum.

(data not shown).

In other experiments, extracts from non-infected cells were sedimented following the same procedure used to isolate SV40 provirions. Material migrating in the 180 S region were electrophoresed on SDS gel and processed as for Figure 1. HMG proteins were not detected, indicating that these proteins seen in the provirion are not due to contamination of large cellular chromatin fragments (data not shown). To rule out the possibility that during the extraction procedure HMGs released from the host chromatin could be nonspecifically adsorbed to the viral particles and eventually detected as such in our assay, we added $^{125}$I-labelled HMGs to infected cells just before homogenization. A sufficient amount of labelled HMGs (2.5 x $10^5$ counts/min, equivalent to 0.5 µg) was added to the cells (2 x $10^7$) to be extracted to assure the detection of contaminating HMGs at levels well below the one found associated with viral particles.

The cell extract was centrifuged in a sucrose gradient of the type used to isolate the SV40 provirions, and the gradient fractions were assayed for radioactivity. The results presented in Figure 4 show that all the radioactivity remained in the top fractions. In particular, the profile in the region

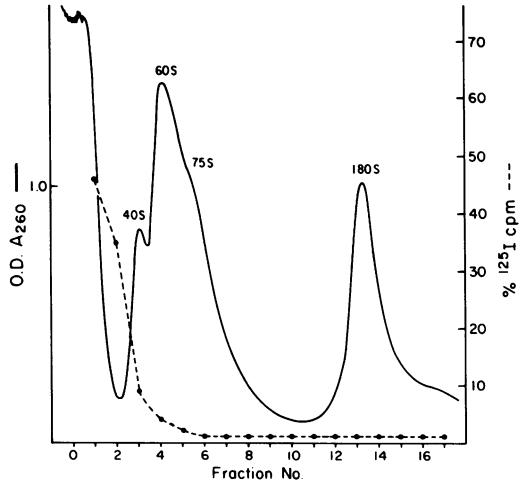


Figure 4. Sucrose gradient fractionation of infected-cell extracts. Extracts from CV-1 cells infected with SV40 were prepared 65 hr post-infection, as described in Methods. Before homogenization, 0.5 µg of $^{125}$I-HMG with a specific activity 5 x $10^5$ cpm/µg were added to 2 x 107 cells. The extracts were centrifuged in a 15-30% sucrose gradient, and the gradient fractions counted for radioactivity.

underlying the 180 S provirions peak was perfectly flat (with the background radioactivity accounting for less than 0.01% of the total). This result indicates that our provirion preparations were not contaminated by adsorbed HMG proteins. The lack of external contamination in the viral particles was also confirmed by means of a very sensitive solid-phase radioimmunoassay. In this test, the antiserum reaction with a fixed amount of plastic-bound HMGs is prevented by the addition of HMG-containing material in scalar amounts. The results of these experiments can be summarized as follows: clear-cut inhibition curves were obtained by adding to the assay provirions pre-incubated with dissolving buffers (containing urea, DTT, 0.1% SDS, and 0.1% Triton-X); this inhibition, however, did not occur by adding to the assay intact provirions (data not shown).

To rule out the possibility of an artifactual redistribution of proteins between SV40 provirion and proteins derived from the host cell, the following experiment was performed. Labelled CV-1 cells were mixed with unlabelled SV40-infected CV-1 cells collected 65 hr post-infection. The cell mixture was homogenized and the cell extract was sedimented under conditions described in Methods. Fractions from the gradient were counted for radioactivity (Figure 5) and the amount of radioactivity detected in the 180 S region was just above the background level and no peak of radioactivity was found in this region. This makes it very unlikely that proteins derived from host cells could be artifactually bound to the provirion during our extraction procedure.

Cross-reactivity between VP3 and HMG-1, HMG-2

An unexpected result in our investigation was the finding that VP3, a virus-coded protein whose specific role is still obscure, reacted with the anti-HMG-1 and anti-HMG-2 sera. The two other SV40 structural proteins, VP2 and VP1, were not reactive. There is no apparent similarity between the primary sequence of VP3 (18) and the partially known sequence of the HMGs (19), but it cannot be excluded that a recognition site, either natural or artificial, may be formed by non-contiguous amino acid residues because of protein folding. This event could well take place during the protein transfer, which, unlike the electrophoresis, is not done under denaturing conditions. A site with this origin is likely to be very sensitive to limited protein digestion. In order to ascertain this sensitivity, we compared the effect of VP3- and HMG-reactivity of protease treatments carried out according to Cleveland *et al.* (17). Figure 6, panel a, shows the electrophoretic patterns of these protein digests which, as expected, were different; panel b of the same

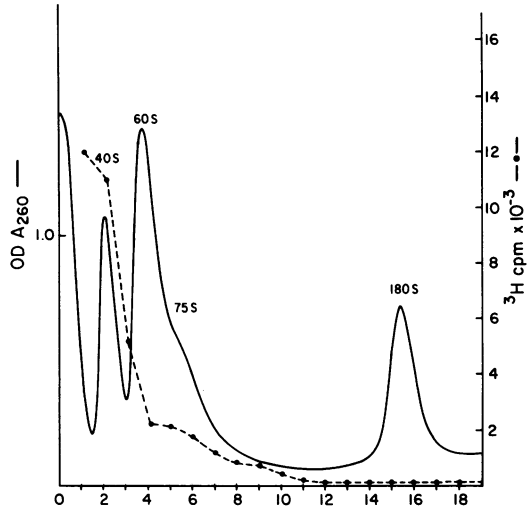


Figure 5. Sucrose gradient fractionation of extracts from uninfected $^{3}H$-labelled CV-1 cells and infected unlabelled CV-1 cells. SV40-infected cells ($10^{7}$) were collected 65 hr post-infection and mixed with $10^{7}$ uninfected cells labelled with $^{3}H$-leucine. The cell mixture was extracted as described in Methods, the extract was centrifuged in a 15-30% sucrose gradient, and the gradient fractions were counted for radioactivity.

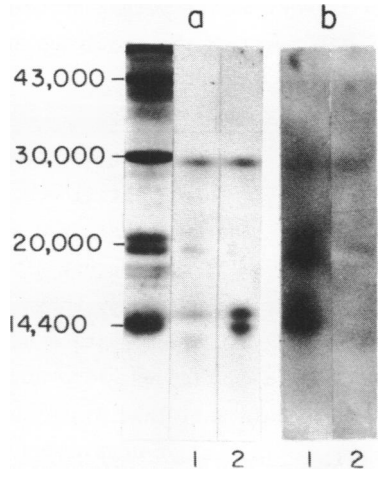


Figure 6. Limited proteolytic digestion of HMG-1, HMG-2, and VP3. Peptides were obtained using V8 protease as the proteolytic enzyme.

a) Stained gel. 1: Peptides of the digested HMG. 2: Peptides of the digested VP3. Molecular weight markers are indicated in the left side of the panel.

b) Autoradiography of the peptides recognized by anti-HMG-2 antiserum.

figure shows the autoradiography of the transferred peptides which reacted with the anti-HMG serum. The weak positivity of VP3 is maintained in a fragment corresponding to about 3/4 of the VP3 m.w., while the fragments in the 14,000 m.w. region, corresponding to about 1/2 molecule are negative; the equivalent half-molecules of the HMGs, on the contrary, are fully reactive. Thus, while the persistence of reactivity in VPs smaller fragments would have made the hypothesis of a conformational reacting site quite unlikely, this result can be said to be at least consistent with it.

## DISCUSSION

The present experiments show that HMG proteins 1 and 2 are associated with the chromatin of SV40 at the provirion stage, but that these proteins, like histone H1 (11), are removed by the time virion maturation is completed.

In order to answer unambiguously the question under study, it was critically important to combine in the same assay a high degree of sensitivity and specificity. This need was satisfied by the protein transfer method (16), in which the resolving power of gel electrophoresis enhanced the sensitivity of protein detection. The antibodies used here did not react with histones and other non-histone chromosomal proteins except HMG-1 and HMG-2, as shown in tests carried out with the same transfer technique. As to the sensitivity of the assay, we calculated that it allowed the detection of much less than one microgram of these proteins.

The possibility that some artifactual adsorption of cellular HMGs to provirions might occur during the cell fractionation was examined carefully, since it has been suggested that HMG proteins are more or less tightly bound to chromatin depending on the cell type (20). This artifact was ruled out by mixing experiments (see Figures 4 and 5) and by a radioimmunoassay in which only disrupted viral particles, but not intact particles, were able to compete with HMGs for antibodies.

A surprising observation coming from our experiments was that one of the virus-coded proteins, VP3, cross-reacted with the anti-HMG-1 and anti-MHG-2 sera. This reaction was rather weak if estimated in terms of relative protein amounts, but practically important, given the respective abundance of HMGs and VP3. In view of the facts that there are no obvious sequence analogies between these molecules, and that VP2 (which shares part of the dequence with VP3) did not react, the most likely explanation of this finding is that VP3, on leaving the gel, renatured in a conformation that produced an artificial recognition site. This hypothesis is consistent with the observation that,

while HMG proteolytic fragments of about half the m.w. still reacted with the antiserum, comparable fragments of VP3 did not. A second, more trivial, possibility was that for some mysterious cause the donor rabbit had come into contact with the virus, but this is rendered very unlikely, among other reasons, by the fact that antiserum did not react with the major viral protein VP1, either in trasnfer assays or in radioimmunoassays carried out under non-denaturing conditions.

The antiserum used in these experiments does not recognize HMG-14 or HMG-17 (Massimo Romani, unpublished observations), therefore we cannot say at present whether these proteins are associated or not with SV40 chromatin; experiments are in progress to answer this question.

The mechanism and the biological meaning of the disappearance from SV40 chromatin of HMG-1 and HMG-2 at the end of morphogenesis remain, like that of histone H1, a matter for speculation. Proteins could be either hydrolyzed or squeezed out. It is not hard to conceive, however, that the viral chromatin, as long as it has to function inside the cell, probably works very efficiently just because it resembles closely the host chromatin. The eventual need to fit into compact symmetrical structures better-suited to travel in the external environment, may require the temporary shedding of some host proteins to accommodate special, virus-specified, packing proteins.

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